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Dennd3 Functions as a Guanine Nucleotide Exchange Factor for Small GTPase Rab12 in Mouse Embryonic Fibroblasts*

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Background: The physiological function of Dennd3, a putative Rab12-GEF, was unknown.
Results: Dennd3 regulates degradation of the amino acid transporter PAT4 through Rab12 activation and also modulates Akt activity.
Conclusion: Dennd3 functions as a Rab12-GEF in MEF cells.
Significance: Our findings provide a novel insight into the cross-talk between an amino acid signaling pathway and a growth factor signaling pathway through Dennd3.

Small GTPase Rab12 regulates mTORC1 (mammalian target of rapamycin complex 1) activity and autophagy through controlling PAT4 (proton/amino acid transporter 4) trafficking from recycling endosomes to lysosomes, where PAT4 is degraded. However, the precise regulatory mechanism of the Rab12-mediated membrane trafficking pathway remained to be determined because a physiological Rab12-GEF (guanine nucleotide exchange factor) had yet to be identified. In this study we performed functional analyses of Dennd3, which has recently been shown to possess a GEF activity toward Rab12 *in vitro*. The results showed that knockdown of Dennd3 in mouse embryonic fibroblast cells caused an increase in the amount of PAT4 protein, the same as Rab12 knockdown did, and knockdown of Dennd3 and overexpression of Dennd3 were found to result in an increase and a decrease, respectively, in the intracellular amino acid concentration. Dennd3 overexpression was also found to reduce mTORC1 activity and promoted autophagy in a Rab12-dependent manner. Unexpectedly, however, Dennd3 knockdown had no effect on mTORC1 activity or autophagy despite increasing the intracellular amino acid concentration. Further study showed that Dennd3 knockdown reduced Akt activity, and the reduction in Akt activity is likely to have canceled out amino acid-induced mTORC1 activation through PAT4. These findings indicated that Dennd3 not only functions as a Rab12-GEF but also modulates Akt signaling in mouse embryonic fibroblast cells.

Rab proteins are a subfamily of small GTPases that function as regulators of membrane traffic in all eukaryotes (1, 2). Thus far, ~60 different members of the Rab family have been identified in mammals, and each member is thought to regulate a specific type of membrane traffic. By cycling between two nucleotide-bound states, a GDP-bound inactive state and a GTP-bound active state, Rabs function as molecular switches. Rabs are generally thought to be activated by specific guanine nucleotide exchange factors (GEFs)[3] and inactivated by specific GTPase-activating proteins (GAPs) (3–5). Thus, the identification and characterization of these Rab regulators, especially of GEFs, is crucial to understanding the spatiotemporal regulation of Rab GTPase activation.

We previously reported finding that Rab12 regulates the trafficking of amino acid transporter PAT4 (proton/amino acid transporter 4)-containing vesicles from recycling endosomes to lysosomes, where PAT4 is degraded (6). We also found that Rab12 modulates the intracellular amino acid concentration, mTORC1 (mammalian target of rapamycin complex 1) activity, and autophagy, a conserved catabolic mechanism in all eukaryotes, through controlling PAT4 degradation (6). However, almost nothing was known in regard to the Rab12-specific GEF. Yoshimura *et al.* (7) recently made a systematic attempt to identify the target/substrate Rab of the DENN (differentially expressed in normal and neoplastic cells) domain-containing proteins (putative Rab-GEFs) in humans and succeeded in demonstrating that DENND3 exerts GEF activity toward Rab12 *in vitro*. However, it remained unclear whether Dennd3 actually functions as a Rab12-GEF *in vivo*.

In the present study we investigated the effect of Dennd3 knockdown and overexpression on PAT4 degradation, mTORC1 activity, and autophagy in MEF cells to elucidate the physiological function of Dennd3 in the regulation of Rab12. The results showed that Dennd3 actually regulates PAT4 degradation and that overexpression of Dennd3 modulates mTORC1 activity and autophagy through Rab12 activation. Unlike Rab12, however, Dennd3 was found to also regulate Akt signaling. Possible mechanisms of the Dennd3-mediated regulation of mTORC1 activity and Akt activity are discussed on the basis of our findings.

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[3] The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; DENN, differentially expressed in normal and neoplastic cells; EGFR, epidermal growth factor receptor; HBSS, Hanks' balanced salt solution; MEF, mouse embryonic fibroblast; mTORC1, mammalian target of rapamycin complex 1; PAT4, proton/amino acid transporter 4; S6K, ribosomal protein S6 kinase; TfR, transferrin receptor.

EXPERIMENTAL PROCEDURES

Materials—Anti-Rab12 rabbit polyclonal antibody and anti-LC3 rabbit polyclonal antibody were prepared as described previously (8, 9). The following primary antibodies were obtained commercially: anti-TfR (transferrin receptor) mouse monoclonal antibody (Zymed Laboratories Inc., San Francisco, CA), anti-EGFR (epidermal growth factor receptor) sheep polyclonal antibody (Fitzgerald Industries International, MA), anti-β-actin mouse monoclonal antibody (Applied Biological Materials, Richmond, BC, Canada), anti-Akt mouse monoclonal antibody (BD Transduction Laboratories), anti-phospho p70S6K1 (Thr-389) antibody, anti-p70S6K antibody, anti-phospho Akt (Thr-308) antibody (Cell Signaling Technology, Danvers, MA), anti-T7 tag mouse monoclonal antibody (Merck), anti-FLAG tag rabbit polyclonal antibody (Sigma), and horseradish peroxidase (HRP)-conjugated anti-HA tag mouse monoclonal antibody (Roche Applied Science). Alexa 488-conjugated secondary antibody was from Invitrogen. A protease inhibitor mixture and phosphatase inhibitor mixture 2 were purchased from Roche Applied Science and Sigma, respectively. Bafilomycin A1 was obtained from Merck KGaA (Darmstadt, Germany).

Plasmid Construction—cDNAs encoding an open reading frame of mouse Rab12 and Dennd3 were prepared as described previously (8, 10). The cDNAs obtained were subcloned into the pMRX-IRES-puro-T7 vector. The pMRX-IRES-puro-T7 vector is a variant of the pMRX-IRES-puro vector (donated by Shoji Yamaoka, Tokyo Medical and Dental University, Tokyo, Japan) (11). pEF-FLAG-Rab12-Q100L (QL) was prepared as described previously (12).

Cell Culture, Transfection, and Infection—Mouse embryonic fibroblasts (MEFs) prepared from 13.5-day mouse embryos were transformed with pEF321-T, an SV40 large T antigen expression vector. The resulting immortalized MEF cell line (simply referred to as MEF cells throughout this paper) was a generous gift of Noboru Mizushima (University of Tokyo, Tokyo, Japan). MEF cells were maintained at 37 °C in DMEM (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS) and antibiotics under 5% CO_2. Starved conditions were achieved by washing the cells once with HBSS (Sigma) and transferring them to HBSS for 1 h. Plat-E cells were donated by Toshio Kitamura (University of Tokyo, Tokyo, Japan). Plat-E cell culture and retrovirus infection were performed essentially as described previously (13). The cells were transfected with plasmid DNAs and siRNAs by using Lipofectamine 2000 and RNAiMAX (Invitrogen), respectively, each according to the manufacturer's instructions.

RNAi—The siRNA against mouse Rab12 and stealth RNAi™ RNA against Dennd3 have been described previously (8). MEF cells were cultured for 72 h after transfection with siRNAs or stealth RNAs.

Quantification of the Intracellular L-Amino Acid Concentration—MEF cells were grown on 3.5-cm dishes. Before harvesting them, the cells were starved for 1 h with HBSS and then restimulated for 15 min with culture medium. The cells were then rinsed 3 times with ice-cold PBS (phosphate-buffered saline) and lysed with the lysis buffer (50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1% Triton X-100, and the protease inhibitor mixture). After normalization of the protein concentration of each sample, the lysates were analyzed by using an L-Amino Acid Quantitation Kit (BioVision, Milpitas, CA) according to the manufacturer's instructions to determine their intracellular L-amino acid concentration.

Immunofluorescence and Image Analyses—Immunostaining was performed essentially as described previously (14). In brief, cultured cells were fixed with 4% paraformaldehyde and stained with specific antibodies. The stained cells were examined for fluorescence with a confocal fluorescence microscope (Fluoview 1000-D; Olympus, Tokyo, Japan) through an objective lens (100× magnification, NA 1.45; Olympus) and with Fluoview software (Version 2.1c; Olympus). For quantitative analysis, images of the cells were captured at random with the confocal microscope, and the number of fluorescent dots (*i.e.* LC3 dots) was counted with ImageJ software (Version 1.42q; National Institutes of Health, Bethesda, MD).

Immunoblotting—Cells were rinsed with ice-cold PBS, scraped, and collected by centrifugation at 4 °C. The cells were then lysed in 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1% Triton X-100, the phosphatase inhibitor mixture 2, and the protease inhibitor mixture, and total cell lysates were obtained by centrifugation at 15,000 × *g* for 10 min at 4 °C. The cell lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blots were then blocked with 1% skim milk in PBS containing 0.1% Tween 20 and incubated with the primary antibodies. Immunoreactive bands were detected by using HRP-conjugated secondary antibodies and enhanced chemiluminescence. The intensity of the immunoreactive bands was quantified with ImageJ software. The blots shown in this study are representative of three independent experiments.

Active Rab12 Pulldown Assay—Lysates from COS7 cells that had been transfected with pEF-T7-RILP-L1 (15) or a control vector were incubated for 1 h at 4 °C with anti-T7 tag antibody-conjugated agarose beads. The beads were washed 3 times with washing buffer (50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.2% Triton X-100, and the protease inhibitor mixture) and then incubated for 1 h at 4 °C with the lysates from Dennd3 knockdown MEF cells or control MEF cells. The beads were then washed 3 times with the washing buffer, and the proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with the antibodies indicated.

Preparation of Total RNA and Reverse Transcription (RT)-PCR—Total RNA was prepared from MEFs by using TRI reagent (Sigma) according to the manufacturer's instructions, and reverse transcription was performed by using a ReverTra Ace-kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The following pairs of primers were used for Gapdh: forward primer, 5′-ATGGTGA AGGTCGGAGTCAA-3′, and reverse primer, 5′-GCCATGTAGACCATGAGGTC-3′; for Dennd3: forward primer, 5′-AAGGCCATGGCGCTGTTG-3′, and reverse primer, 5′-TGCAGGATCCAGGCGCCC-3′. cDNAs were amplified by PCR with Ex Taq (Clontech-Takara Bio Inc., Shiga, Japan) and by performing 20 cycles (for Gapdh) or 25 cycles (for Dennd3) of denaturation at 98 °C for 10 s, annealing at 55 °C for 0.5 min, and extension at 72 °C for 1 min.

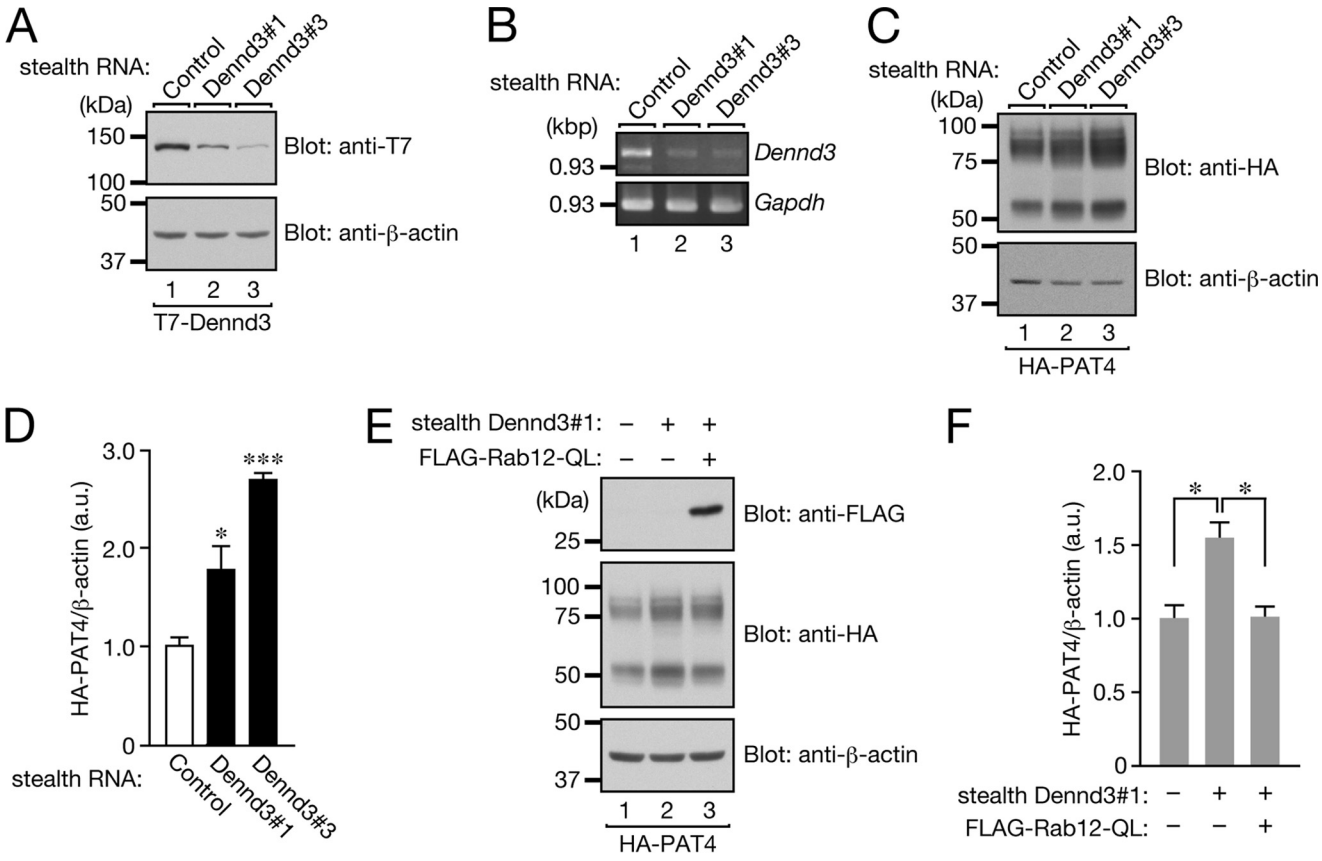


FIGURE 1. **Dennd3 regulates PAT4 degradation as an upstream activator of Rab12 in MEF cells.** *A*, the efficiency of stealth RNAs against Dennd3 is shown. MEF cells stably expressing T7-Dennd3 were transfected with control or *Dennd3* stealth RNAs. Cell lysates were analyzed by immunoblotting with the antibodies indicated. *B*, RNAi-mediated knockdown of Dennd3 in wild type MEF cells as demonstrated by RT-PCR. *Gapdh* was used as an internal control. The size of the molecular weight markers (bp) is shown on the *left*. *C*, Dennd3 knockdown resulted in an increase in the amount of PAT4 protein. MEF cells stably expressing HA-PAT4 were transfected with control or *Dennd3* stealth RNAs. Cell lysates were analyzed by immunoblotting with the antibodies indicated. *D*, quantification of the results shown in *C*. *E*, the effect of Dennd3 knockdown on the degradation of PAT4 protein was canceled out by simultaneous expression of a constitutive active mutant of Rab12 (Rab12-QL). MEF cells stably expressing HA-PAT4 were transfected with control or *Dennd3* stealth RNAs. One day after stealth RNA transfection, the cells were transfected with pEF-BOS control vector or pEF-FLAG-Rab12-QL, and 2 days after plasmid transfection cell lysates were analyzed by immunoblotting with the antibodies indicated. The positions of the molecular mass markers (in kDa) are shown on the left in *A*, *C*, and *E*. *F*, quantification of the results shown in *E*. *, $p < 0.05$; ***, $p < 0.005$. *a.u.*, arbitrary units.

L-Amino Acid Treatment—MEF cells stably expressing T7-Dennd3 were treated with 0.8 mM Leu, 0.1 mM Pro, 0.08 mM Trp, or 0.1 mM Ala in the culture medium for 30 min, after which cell lysates were immediately prepared and analyzed by immunoblotting as described above.

Statistical Analysis—The statistical analyses were performed by using Student's unpaired *t* test, and *p* values <0.05 were considered statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

RESULTS

Dennd3 Regulates PAT4 Degradation and the Intracellular Amino Acid Concentration—To determine whether Dennd3 acts as an upstream activator of Rab12 *in vivo*, we first examined the effect of Dennd3 knockdown on PAT4 degradation because Rab12 knockdown has been shown to increase the amount of PAT4 protein in MEF cells (6). The results of an immunoblot analysis of MEF cells stably expressing HA-PAT4 showed that knockdown of endogenous Dennd3 in the cells with two independent specific stealth RNAs (Fig. 1, *A* and *B*) resulted in a significant increase in the amount of PAT4 protein (Fig. 1, *C* and *D*). Moreover, the effect of Dennd3 knockdown on the amount of PAT4 protein was completely canceled out by simultaneous overexpression of a constitutive active mutant of Rab12 (Rab12-QL), *i.e.* by forced activation of Rab12 (Fig. 1, *E* and *F*), suggesting that Dennd3 knockdown in MEF cells causes inactivation of Rab12, which in turn leads to inhibition of PAT4 degradation. To biochemically determine whether Rab12 is actually inactivated in Dennd3 knockdown cells, we performed active Rab12 pulldown assays by using beads coupled with RILP-L1, an active Rab12-binding protein (15). The results showed that Dennd3 knockdown significantly decreased the amount of the active form of Rab12 in the Dennd3 knockdown cells (Fig. 2, *A* and *B*). These results indicated that Dennd3 regulates PAT4 degradation in MEF cells by functioning as an upstream activator of Rab12.

We next examined the effect of Dennd3 knockdown on the intracellular amino acid concentration of MEF cells because Rab12 knockdown has been shown to result in an increased intracellular L-amino acid concentration (6). As expected, the intracellular L-amino acid concentration was significantly higher in the Dennd3 knockdown cells than in the control cells (Fig. 3), consistent with the increased amount of PAT4 protein (Fig. 1*D*). Thus, Dennd3 is likely to modulate the intracellular

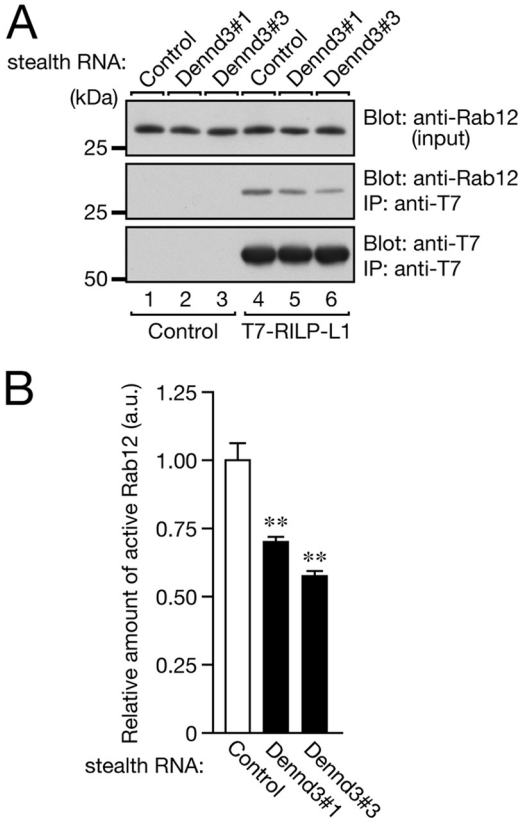


FIGURE 2. **Effect of Dennd3 knockdown on the level of active Rab12.** *A*, cell lysates from control or Dennd3 knockdown MEF cells were incubated with beads coupled with T7-RILP-L1, a trapper of active Rab12 (15). *Input* and immunoprecipitates (*IP*) were analyzed by immunoblotting with the antibodies indicated. The positions of the molecular mass markers (in kDa) are shown on the *left*. *B*, quantification of the results shown in *A*. Note that Dennd3 knockdown caused a significant reduction in the amount of endogenous active Rab12. **, $p < 0.01$. *a.u.*, arbitrary units.

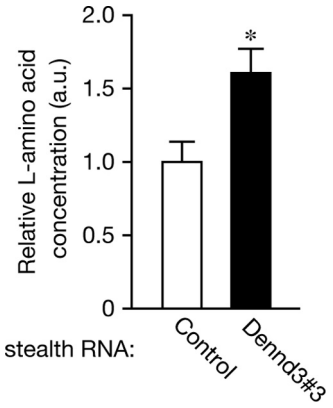


FIGURE 3. **Effect of Dennd3 knockdown on the intracellular L-amino acid concentration.** Control and Dennd3 knockdown cells were analyzed with an L-Amino Acid Quantitation kit (see "Experimental Procedures" for details). Note that knockdown of Dennd3 resulted in an increase in the amount of intracellular L-amino acid concentration. *, $p < 0.05$. *a.u.*, arbitrary units.

amino acid concentration through controlling the degradation of PAT4, the same as Rab12 does.

Dennd3 Knockdown Does Not Affect Autophagy—Induction of autophagy is known to be regulated by intracellular nutrients, including by amino acids and growth factors (16, 17), and since we had recently shown that the efficiency of autophagy induction was reduced in Rab12 knockdown MEF cells because of the increased amount of intracellular L-amino acids entering through PAT4 (6), we next investigated the impact of Dennd3 knockdown on autophagy by means of three independent approaches (Fig. 4) (see Ref. 18 for details). Unexpectedly, however, the results showed that Dennd3 knockdown had no effect at all on the autophagic flux or the degradation of p62 protein, a specific substrate of autophagy (Fig. 4, *A* and *B*), and there was only a small reduction in the number of dots that were positive for LC3, an autophagosome marker (to ~70–80% of the number in the control cells (Fig. 4, *C* and *D*). These results were in sharp contrast to our previous findings of a clear decrease in the number of LC3 dots, delayed autophagic flux, and increased amount of p62 protein in Rab12 knockdown cells in comparison with the control cells (*i.e.* typical autophagy inhibition phenotypes) (6). Because in contrast to Rab12 knockdown Dennd3 knockdown had little effect on autophagy, unlike in MEF cells (Fig. 4), we hypothesized that Dennd3 has an additional function other than Rab12 activation during induction of autophagy.

Dennd3 Knockdown Decreases Akt Activity without Affecting mTORC1 Activity—The results of the above experiments indicated that even though Dennd3 knockdown results in an increase in the intracellular amino acid concentration (Fig. 3), it had hardly any effect on autophagy (Fig. 4). To resolve this discrepancy, we examined mTORC1 activity in Dennd3 knockdown cells because mTORC1 negatively regulates autophagy in response to amino acids, growth factors, and/or cellular energy (16, 17, 19–24). Interestingly, Dennd3 knockdown had no effect at all on the phosphorylation level of ribosomal protein S6 kinase (S6K), a specific substrate of mTORC1 (Fig. 5*A*, *second panel*, and *B*). To our surprise, however, it clearly decreased the phosphorylation level of Akt (Fig. 5*A*, *fourth panel*, and *B*) even though we had found earlier that Rab12 knockdown increases mTORC1 activity without affecting Akt activity (6). In addition, we found that the amount of EGFR protein, which is known to activate Akt, in the Dennd3 knockdown cells was reduced to approximately half the amount of EGFR protein in the control cells (Fig. 5, *A* and *B*), suggesting that Dennd3 knockdown reduces Akt activity through down-regulation of the EGFR-Akt pathway. These findings led us to hypothesize that Dennd3 knockdown not only up-regulates mTORC1 activity through the increased intracellular amino acid concentration (*i.e.* the amino acid-mTORC1 pathway) (Fig. 3) but also down-regulates the Akt-mTORC1 pathway and that its effects on the two pathways cancel each other out, thereby resulting in no apparent change in mTORC1 activity (Fig. 5, *A* and *B*).

Because according to this hypothesis, forced activation of Akt in Dennd3 knockdown cells should result in stronger activation of mTORC1 than in control cells, we tested it by stimulating Dennd3 knockdown cells with insulin or EGF, either of which forcibly activates the Akt-mTORC1 pathway (Fig. 5, *C* and *D*). The results showed that insulin stimulation or EGF stimulation restored Akt activity (*i.e.* phosphorylation of Akt) in Dennd3 knockdown cells to the same level as in the control cells (Fig. 5*C*, *middle panel*, and *D*, *fourth panel*, *lanes 4–6*) and increased mTORC1 activity (*i.e.* the phosphorylation level of S6K) (Fig. 5, *C* and *D*, *top panels*, *lanes 4–6*), thereby support-

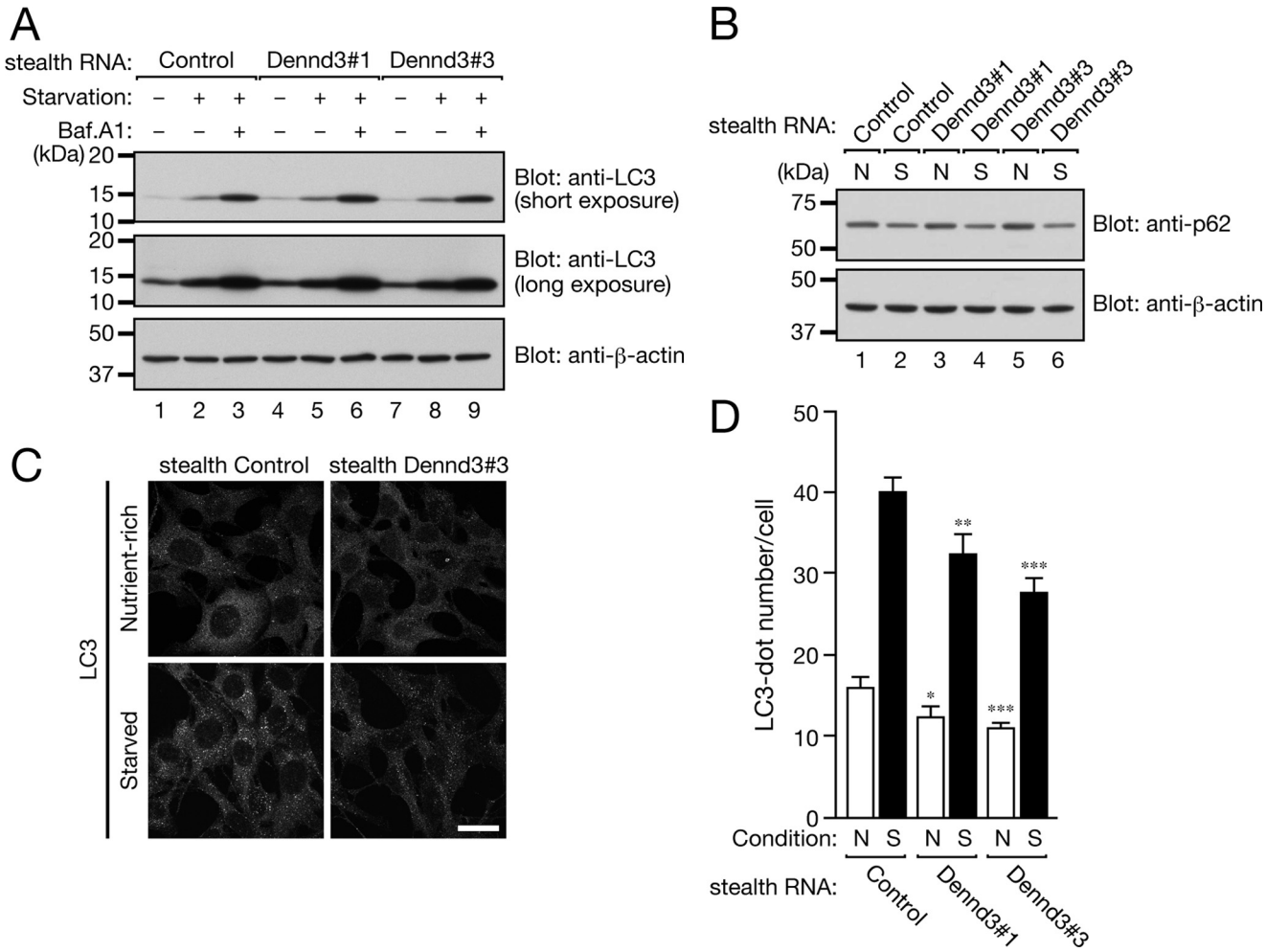


FIGURE 4. **Effect of Dennd3 knockdown on autophagy.** *A,* Dennd3 knockdown had no clear effect on autophagic flux. Control and Dennd3 knockdown MEF cells were cultured for 1 h under nutrient-rich (*N*) or starved (*S*) conditions in the absence or presence of 100 nM bafilomycin A1 (*BafA1*). Cell lysates were analyzed by immunoblotting with the antibodies indicated. *B*, Dennd3 knockdown had no clear effect on p62 degradation. Control and Dennd3 knockdown MEF cells were cultured under nutrient-rich or starved conditions for 2 h. Cell lysates were analyzed by immunoblotting with the antibodies indicated. The positions of the molecular mass markers (in kDa) are shown on the *left* in *A* and *B*. *C*, Dennd3 knockdown resulted in a slight decrease in the number of LC3-positive dots. Control and Dennd3 knockdown MEF cells were cultured for 1 h under nutrient-rich or starved conditions, fixed, and then immunostained with anti-LC3 antibody. *Scale bar*, 20 μm. *D,* quantification of the results shown in *C*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. *a.u.*, arbitrary units.

ing our hypothesis. Intriguingly, when the cells were cultured in DMEM without FBS (Fig. 5*C*, *top panel*, and *D, second panel*, *lanes 1–3*), Dennd3 knockdown also resulted in an increase in the phosphorylation level of S6K, suggesting that Dennd3 is capable of modulating mTORC1 activity independent of Akt activity. These results indicated that Dennd3 not only regulates mTORC1 activity as a Rab12-GEF but also regulates Akt activity in a Rab12-independent manner.

Overexpression of Dennd3 Reduces mTORC1 Activity in a Rab12-dependent Manner—Finally, we examined the effect of overexpression of Dennd3 on mTORC1 activity and autophagy. To do so, we generated MEF cells stably expressing T7-Dennd3 (Fig. 6*B*, *top panel*). When we measured the intracellular L-amino acid concentration, we found that, in contrast to the Dennd3 knockdown cells, the Dennd3-overexpressing cells contained a reduced intracellular L-amino acid concentration (Figs. 3 and 6*A*) and that overexpression of Dennd3 had reduced mTORC1 activity (Fig. 6, *B*, *third panel*, and *C*), the same as overexpression of Rab12-QL had (6). Because the effect of Dennd3 overexpression on mTORC1 activity was canceled out by exposure of the Dennd3-overexpressing cells to proline (Fig. 6, *D* and *E*), which has been reported to be a high affinity substrate of PAT4 in *Xenopus* oocytes (25), the PAT4 degradation that results from overexpression of Dennd3 is likely to contribute to the reduced mTORC1 activity. Moreover, it should be noted that overexpression of Dennd3 had no effect on Akt activity (Fig. 6*B*, *fifth panel*, and *C*) even though Dennd3 knockdown reduced it (Fig. 5*A*, *fourth panel*), and Dennd3 overexpression increased the amount of EGFR protein (Fig. 6*B*, *seventh panel*, and *C*). This unexpected finding may have been due to Akt activity in the MEF cells having become saturated under our culture conditions irrespective of Dennd3 overexpression. In addition, the amount of TfR protein, another Rab12-cargo (8), was lower in the Dennd3-overexpressing cells (Fig. 6*B*, *second panel*) and higher in the Dennd3 knockdown cells (Fig. 5*A*, *top panel*), respectively, thereby strongly supporting our hypothesis that Dennd3 functions as a physiological Rab12-GEF in MEF cells. Moreover, overexpression of Dennd3 resulted in an increase in the number of LC3-dots (Fig. 6, *F* and *G*) and amount of LC3-II protein, a lipidated form of LC3 spe-

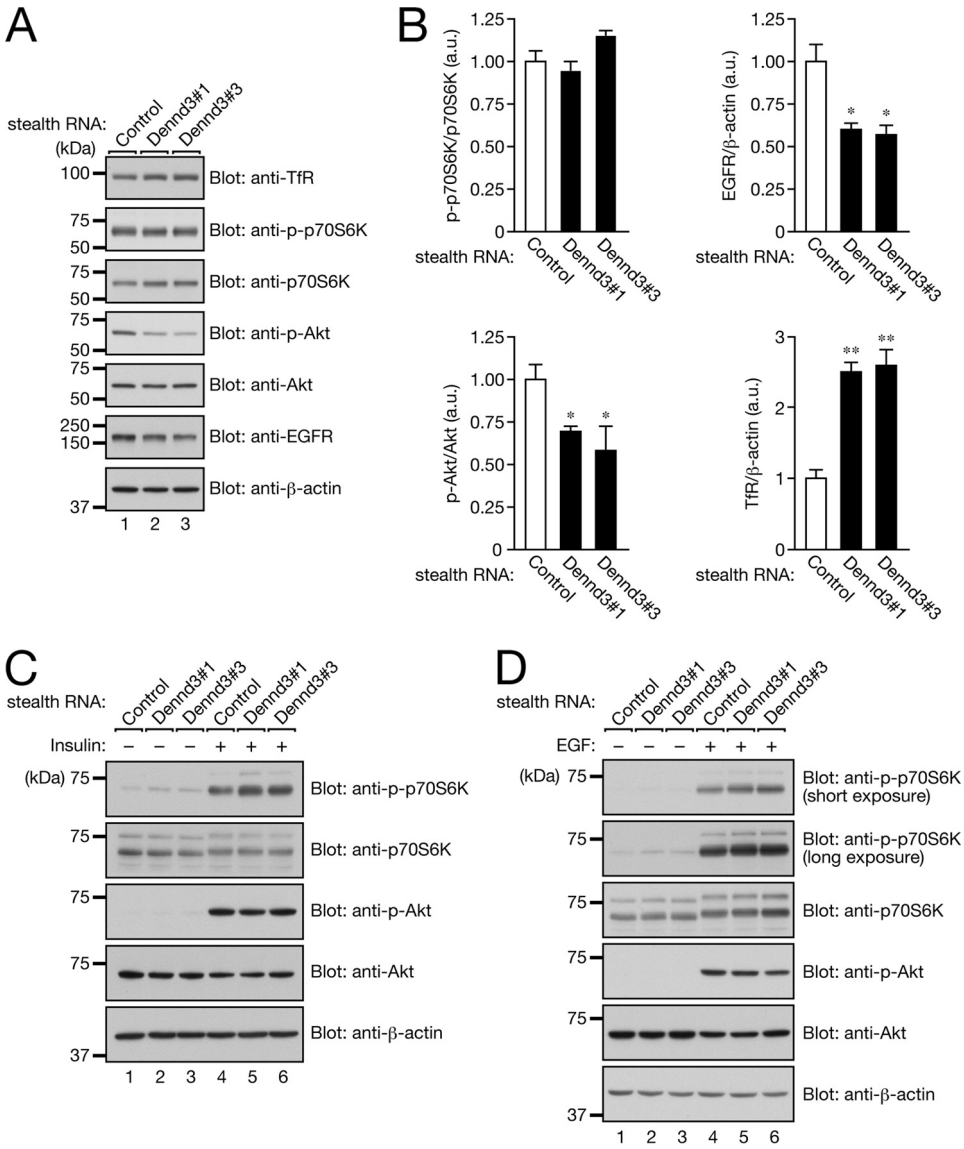


FIGURE 5. **Effect of Dennd3 knockdown on nutrient signals.** *A*, Dennd3 knockdown resulted in a decrease in Akt activity. Control and Dennd3 knockdown MEF cells were starved for 1 h with HBSS and then restimulated for 15 min with culture medium before lysis. Cell lysates were analyzed by immunoblotting with the antibodies indicated. Note that Dennd3 knockdown had no effect on the phosphorylation level of S6K (p70S6K; *second panel*) but that a dramatic decrease in Akt activity (*i.e.* p-Atk) was observed (*fourth panel*). *B*, quantification of the results shown in *A*. *, $p < 0.05$; **, $p < 0.01$. *a.u.*, arbitrary unit. *C* and *D*, insulin stimulation (*C*) or EGF stimulation (*D*) increased mTORC1 activity in Dennd3 knockdown cells in comparison with the control cells. Control and Dennd3 knockdown MEF cells were starved for 12 h with DMEM without FBS and then stimulated for 1 h with 250 nM insulin or for 30 min with 100 ng/ml EGF. Cell lysates were analyzed by immunoblotting with the antibodies indicated. It should be noted that insulin stimulation or EGF stimulation restored Akt activity in Dennd3 knockdown cells to the same level as in the control cells (*middle panel* in *C* and *fourth panel* in *D*, *lanes 4–6*) and increased mTORC1 activity (*i.e.* p-70S6K) (*top panels* in *C* and *D*, *lanes 4–6*). Increased phosphorylation of S6K was also observed in Dennd3 knockdown cells cultured in DMEM without FBS in the absence of insulin (*top panel* in *C*, *lanes 1–3*) or EGF (*second panel* in *D*, *lanes 1–3*). The positions of the molecular mass markers (in kDa) are shown on the *left* in *A*, *C*, and *D*.

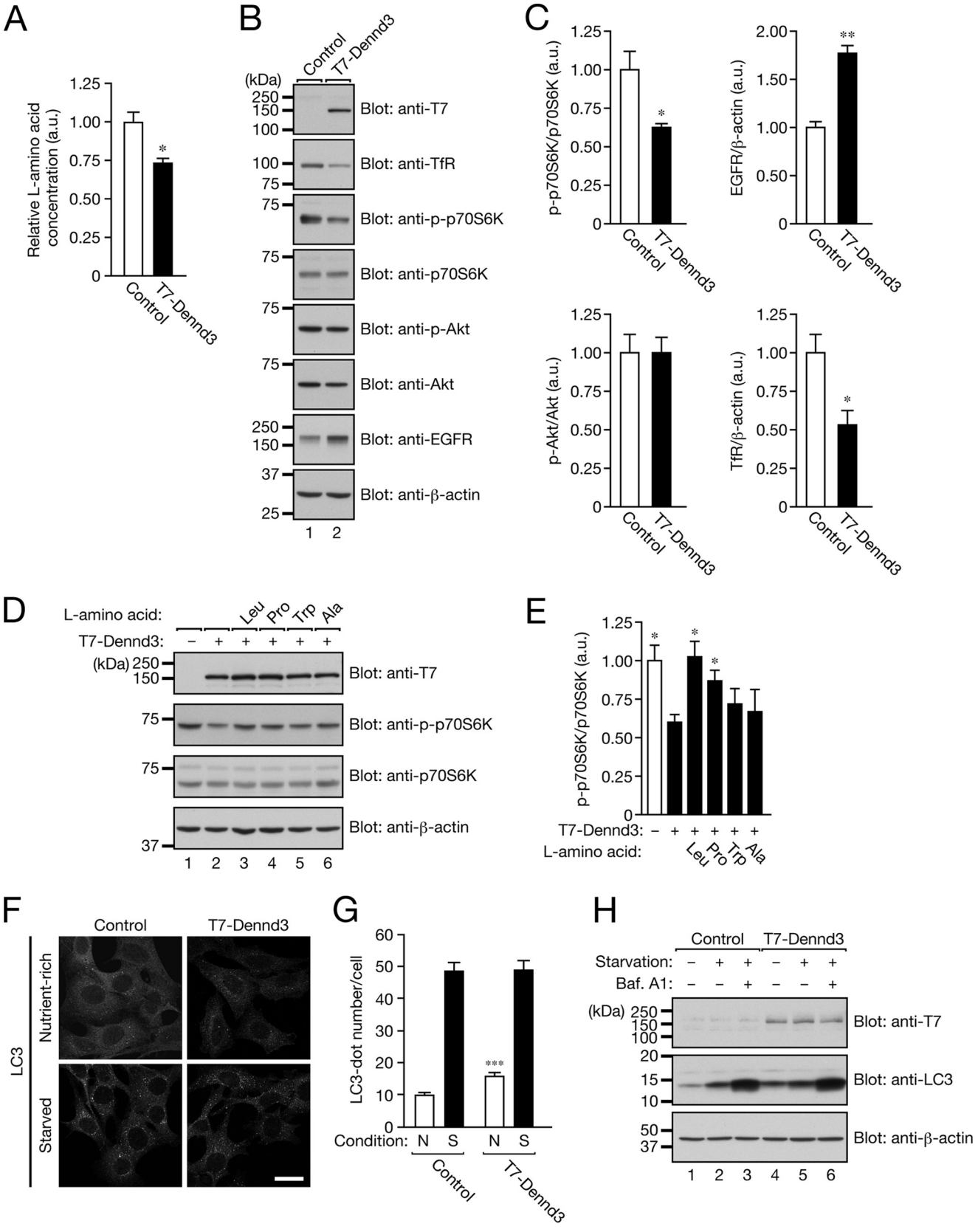
cifically associated with autophagosomes (18), under nutrient-rich conditions alone (Fig. 6*H*, *middle panel*, *lanes 1* and *4*).

To further determine whether or not the effects of overexpression of Dennd3 on mTORC1 activity and TfR degradation are dependent on Rab12, we knocked down endogenous Rab12 with a specific siRNA in control cells and Dennd3-overexpressing cells and analyzed their lysates by immunoblotting. The results showed that the reduction in mTORC1 activity and amount of TfR protein by Dennd3 overexpression was completely canceled out by simultaneous Rab12 knockdown (Fig. 7*A*, *third* and *fourth panels*, *lanes 2* and *4*, and *B*). Moreover, the increase in number of LC3-dots in Dennd3-overexpressing cells under nutrient-rich conditions was also canceled out by Rab12 knockdown (Fig. 7*C*). Taken together, these results indicated that Dennd3 functions as an upstream activator of Rab12 in MEF cells.

DISCUSSION

We previously reported finding that Rab12 modulates mTORC1 activity and autophagy through regulation of the membrane trafficking pathway from recycling endosomes to lysosomes, where PAT4 is degraded (6). In the present study we investigated the physiological function of Dennd3, a putative Rab12-GEF (7), in MEF cells, and we obtained findings that indicated Dennd3 actually functions as an upstream regulator of Rab12. First, we found that Dennd3 knockdown resulted in

an increase in the amount of PAT4 protein, the same as Rab12 knockdown did (6), and that this effect was canceled out by simultaneous expression of the constitutive active Rab12-QL mutant (Fig. 1). Second, we found that knockdown of Dennd3 resulted in an increase in the intracellular L-amino acid concentration, the same as Rab12 knockdown increased it (6), and that



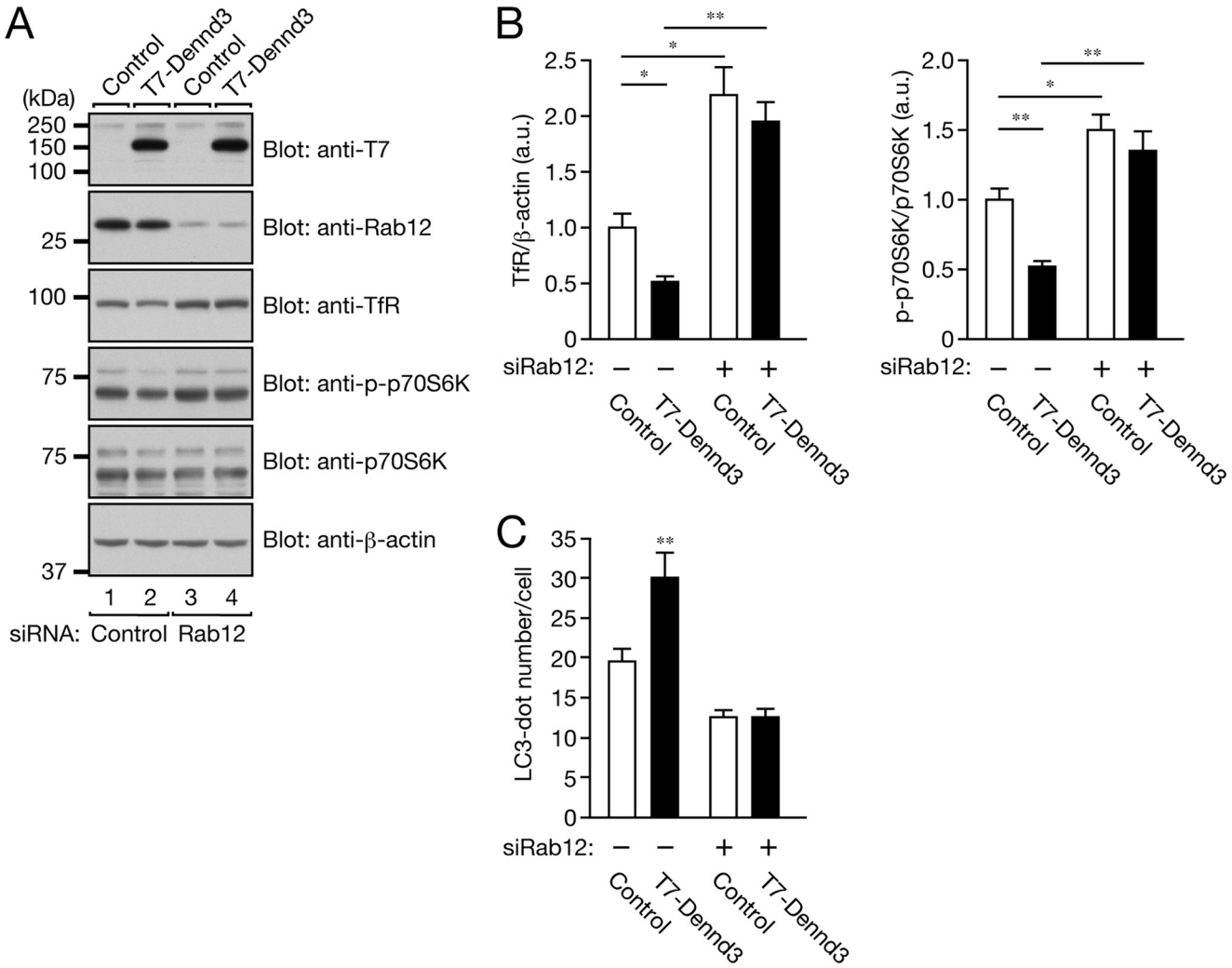


FIGURE 7. **Overexpression of Dennd3 modulates mTORC1 activity and autophagy in a Rab12-dependent manner.** *A,* the effect of Dennd3 expression on mTORC1 activity was canceled out by knockdown of Rab12. Control and T7-Dennd3-expressing MEF cells were transfected with control siRNA or *Rab12* siRNA, and cell lysates were analyzed by immunoblotting with the antibodies indicated. The positions of the molecular mass markers (in kDa) are shown on the *left*. *B*, quantification of the results shown in *A*. *, $p < 0.05$; **, $p < 0.01$. *a.u.*, arbitrary unit. *C,* overexpression of Dennd3 resulted in an increase in the number of LC3-dots in a Rab12-dependent manner. Control and T7-Dennd3-expressing MEF cells were cultured as in *A,* fixed, and then immunostained with anti-LC3 antibody.

overexpression of Dennd3 decreased the intracellular L-amino acid concentration (Figs. 3 and 6*A*). Third, we found that overexpression of Dennd3 reduced mTORC1 activity and promoted autophagy in a Rab12-dependent manner under nutrient-rich conditions alone (Figs. 6, *B*, *C*, and *F*, and 7). In contrast to Rab12 knockdown (6), however, Dennd3 knockdown had hardly any effect on autophagy or mTORC1 activity despite increasing the intracellular amino acid concentration (Figs. 3 and 4). Thus, Dennd3 is not simply a Rab12-GEF but is likely to have an additional function in the regulation of mTORC1 activity and autophagy that is Rab12-independent. We found that Dennd3 is actually involved in the regulation of the amount of EGFR protein, which activates the Akt-mTORC1 pathway (Fig. 5, *C* and *D*), and that Dennd3 knockdown was followed by a reduction in Akt activity, which in turn resulted in the decreased mTORC1 activity (Fig. 5*A*).

FIGURE 6. **Effect of Dennd3 overexpression on mTORC1 activity and autophagy.** *A*, Dennd3 overexpression resulted in a reduction in the intracellular L-amino acid concentration. Lysates of control and T7-Dennd3-expressing cells were analyzed with an L-Amino Acid Quantitation kit. *, $p < 0.05$. *a.u.*, arbitrary unit. *B*, Dennd3 overexpression resulted in a reduction in the amount of TfR protein and mTORC1 activity independent of Akt activity. Control and T7-Dennd3-expressing MEF cells were starved for 1 h with HBSS and then restimulated for 15 min with culture medium. Cell lysates were analyzed by immunoblotting with the antibodies indicated. *C*, quantification of the results shown in *B*. *, $p < 0.05$; **, $p < 0.01$. *D*, addition of L-amino acids partially restored mTORC1 activity. Control and T7-Dennd3-expressing MEF cells were treated for 30 min with the L-amino acids indicated, and lysates of the cells were analyzed by immunoblotting with the antibodies indicated. *E*, quantification of the results shown in *D*. *, $p < 0.05$. *F,* Dennd3 expression resulted in an increase in the number of LC3-positive dots under nutrient-rich conditions. Control and T7-Dennd3-expressing MEF cells were cultured under nutrient-rich (*N*) or starved (*S*) conditions, fixed, and then immunostained with anti-LC3 antibody. *Scale bar*, 20 μm. *G,* quantification of the results shown in *F*. ***, $p < 0.005$. *H,* Dennd3 expression had virtually no effect on autophagic flux. Control and T7-Dennd3-expressing MEF cells were cultured for 1 h under nutrient-rich or starved conditions in the absence or presence of 100 nM bafilomycin A1 (*BafA1*). Cell lysates were analyzed by immunoblotting with the antibodies indicated. Note that the amount of LC3-II in T7-Dennd3-expressing cells increased only under nutrient-rich conditions, in comparison with the control cells (*middle panel*, compare *lanes 1* and *4*). The positions of the molecular mass markers (in kDa) are shown on the *left* in *B*, *D*, and *H* .

The results of our study raise several intriguing questions. First, what is the mechanism by which Dennd3 regulates the activation of Rab12? One possibility is posttranslational modification of Dennd3, because the function of some Rab regulators (GEFs and GAPs) is known to be regulated by phosphorylation or ubiquitination (26–29). For example, insulin-elicited active Akt phosphorylates TBC1D4/AS160 and suppresses its GAP activity toward Rab10, thereby leading to an increase in the GTP-bound form of Rab10, which ultimately promotes trafficking of Glut4 (glucose transporter 4)-containing vesicles to the plasma membrane (29). Because Dennd3 and Rab12 modulate the intracellular amino acid concentration by controlling PAT4 degradation (Figs. 3 and 6*A*), presumably a protein involved in the amino acid sensing mechanism modifies the Dennd3 GEF function that activates Rab12, thereby leading to a decrease in the PAT4 protein level and the intracellular amino acid concentration to the basal level. Extensive research will be necessary to determine whether treatment of MEF cells with an excess of amino acids, specifically, proline, which has been found to be a high affinity substrate of PAT4 (25), will alter posttranslational modification of Dennd3 and Rab12-GEF activity. Despite our unexpected discovery that Dennd3 is involved in the Akt-mTORC1 pathway (Fig. 5*A*), Akt itself does not appear to be a modifier of Dennd3, because our results showed that Dennd3 knockdown resulted in an increase in mTORC1 activity under both Akt-inactive and -hyperactive conditions (Fig. 5, *C* and *D*).

The second intriguing question is how Dennd3 affects Akt activity. We can think of two possible mechanisms. One possible mechanism is the negative feedback mechanism on Akt signaling by mTORC1 and S6K. Several groups have recently reported that mTORC1 and S6K mediate phosphorylation of insulin receptor substrate 1 (IRS1), which is a negative regulator of insulin/IGF-1 signaling, and growth factor receptor-bound protein 10 (Grb10), which is a negative regulator of growth factor signaling, and that these effects suppress the activity of phosphatidylinositol 3-kinase and mTORC2, an upstream activator of Akt (30–33). More recently, S6K has been shown to phosphorylate Sin1, a component of mTORC2, which causes it to dissociate from mTORC2 and suppress mTORC2 activity independent of IRS1 and Grb10 (34). The results of these studies suggest that Dennd3 knockdown initially may increase mTORC1 activity and S6K activity through the increased intracellular amino acid concentration, but that negative regulation of Akt signaling is subsequently induced during cell culture of Dennd3 knockdown cells. However, there is evidence that no such feedback mechanism may actually exist, because Rab12 knockdown also resulted in increases in the intracellular amino acid concentration and mTORC1 activity independent of Akt activity in Rab12-knockdown cells (6). Furthermore, although negative feedback regulation of Akt by insulin or growth factor stimulation has already been demonstrated, it is unknown whether amino acids also induce such a negative feedback mechanism.

The second possible mechanism is that Dennd3 has at least two functions, *i.e.* as a Rab12-GEF and as a positive regulator of Akt signaling. Because Dennd3 contains a DENN domain in its N-terminal region and a WD40 repeat in its C-terminal region (7, 35) and WD40 repeats often function as a protein-protein interaction site, Dennd3 may bind certain proteins involved in the regulation of Akt signaling. We favored this latter possibility because we found that knockdown and overexpression of Dennd3 reduced and increased, respectively, the amount of EGFR protein (Figs. 5, *A* and *B*, and 6, *B* and *C*) that is known to activate Akt. Thus, it is highly possible that Dennd3 modulates Akt activity through EGFR degradation or translation independent of Rab12 activation.

Why does a single protein have two independent roles in mTORC1 regulation? The results of our Dennd3 knockdown experiments showed that Dennd3 acts as both a negative regulator of amino acid-mTORC1 signaling and a positive regulator of growth factor-Akt signaling. Interestingly, Naito *et al.* (36) have recently reported that the mTORC1-autophagy pathway is differentially regulated by insulin and amino acids in a tissue-dependent manner, because they found that insulin treatment increased mTORC1 activity to a greater extent in skeletal muscles than in liver, whereas amino acid treatment increased mTORC1 activity to a greater extent in liver than in skeletal muscles. It is, therefore, tempting to speculate that the level of Dennd3 expression is differentially regulated in the two tissues, *e.g.* Dennd3 expression in liver may be weaker than in skeletal muscles, thereby resulting in greater amino acid sensitivity in liver because of a higher concentration of an increased amount of PAT4 protein, whereas Akt activity is suppressed. An investigation of the precise tissue distribution of Dennd3 will be necessary to answer this question.

In summary, we have demonstrated that Dennd3 functions as a Rab12-GEF in MEF cells. We also unexpectedly discovered that Dennd3 not only regulates Rab12-dependent PAT4 degradation but also modulates Akt signaling in MEF cells. Further studies on the function of Dennd3 may provide new insights into the regulatory mechanism of amino acid- and/or growth factor-mediated mTORC1 signaling.

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